Synthesis of Caged Q-Rhodamine and Fluorescein Conjugated to the Antibody 9EG7

Olga Vasalatiy and Shannon Cofiell

Imaging Probe Development Center, National Heart, Lung, and Blood Institute, National Institutes of Health, 9800 Medical Center Drive, Rockville, MD 20850, ipdc@nhlbi.nih.gov

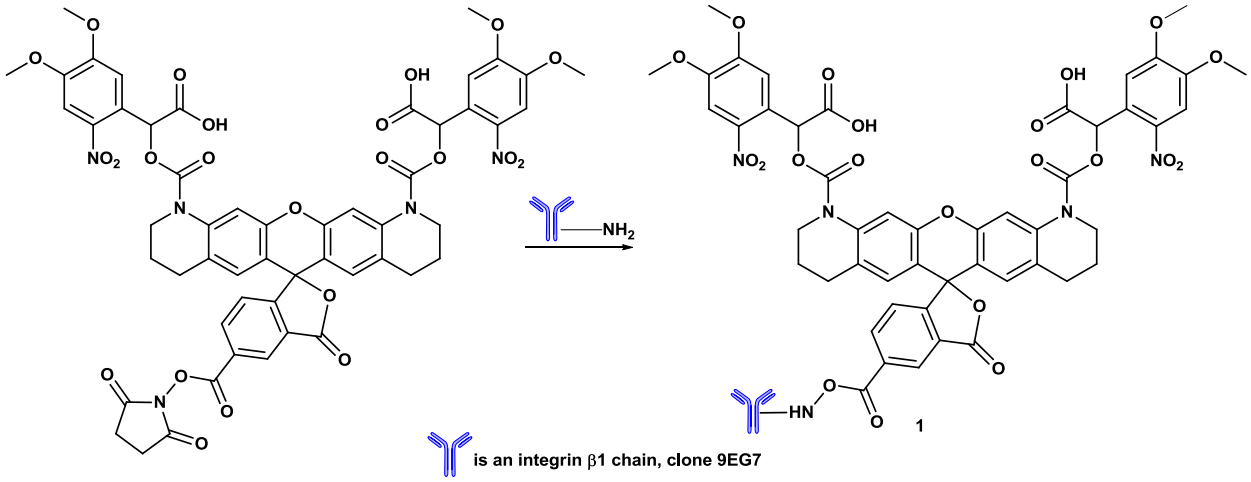
Background

During migration cells send out protrusions to explore the environment. While many cues that direct migration have been identified, mechanisms which govern cell response to the environment are still unclear. Understanding these fundamental mechanisms could be key for new therapeutic treatments of cancer[1]. Sideways polymerization of actin at the edge of cell protrusions have been recently indentified[2]. These polymerization clusters are transmembrane adhesion receptors that bind to the extracellular environment or matrix. Integrin adhesion molecules do not interact with actin directly but rather employ linker molecules that assemble into scaffolds that enable actin connection to integrins. This dynamic process is one of the cells mechanisms to search for suitable sites of attachment to the extracellular environment. However, detection of conformational changes to adhesion receptors during protrusions is limited by resolution of conventional microscopy. A new superresolution technique, PhotoActivation Localization Microscopy (PALM) allows for identification of the position of a specific protein with a 100-fold increase in accuracy in comparison to conventional microscopy, by selective excitation of photoactivatable species[3]. A specific antibody (9EG7) that binds in response to a conformational change in adhesion receptors is to be tagged with a photoactivatable dye for superresolution PALM microscopy for detection studies of conformational form changes of adhesion receptors.

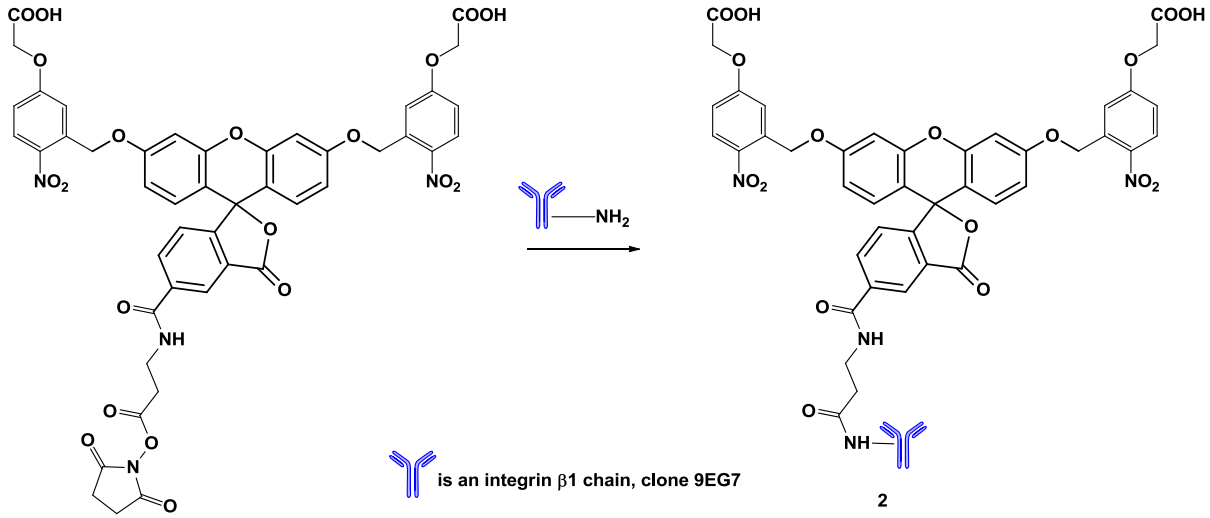
Chemistry

The conjugations of double caged Q-rhodamine dye and 5-carboxymethoxy-2-nitrobenzyl (CMNB)-caged carboxyfluorescein dye to a 9EG7 (purified rat anti-mouse CD29) antibody are shown in schemes 1 and 2, respectively.

Scheme 1. Conjugation of Double Caged Q-Rhodamine dye to a 9EG7 antibody



Scheme 2. Conjugation of 5-carboxymethoxy-2-nitrobenzyl (CMNB)-Caged Carboxyfluorescein dye to a 9EG7 antibody



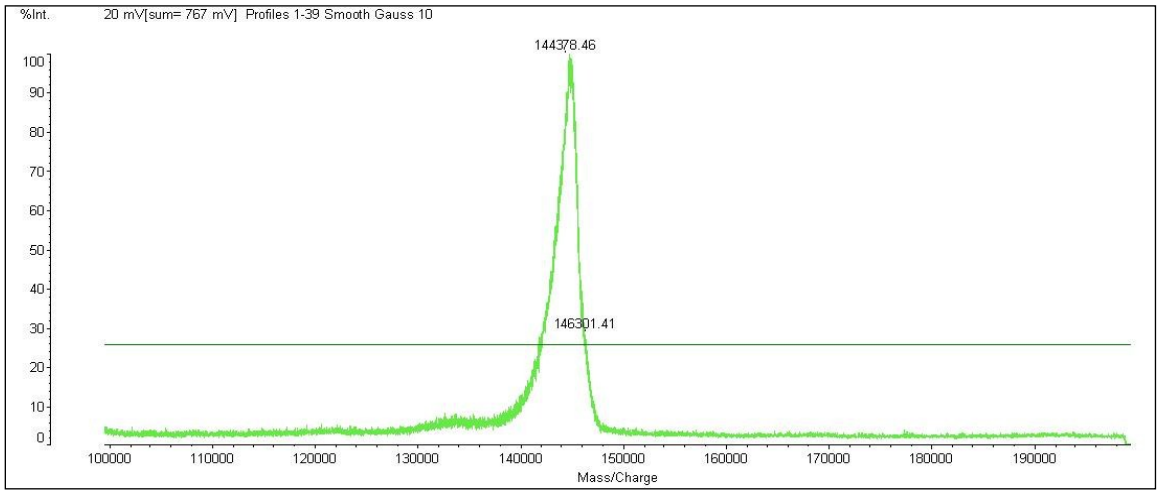


Figure 1. MALDI-TOF of 9EG7 antibody

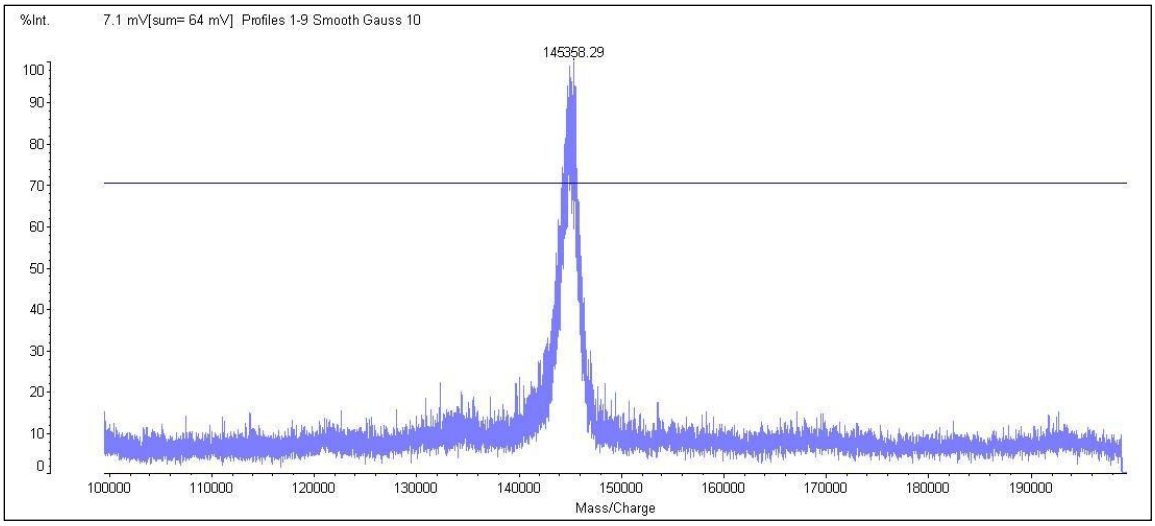


Figure 2. MALDI-TOF data of Q-Rhodamine 9EG7 bioconjugate. The MW is 980 units higher than the unconjugated MAb.

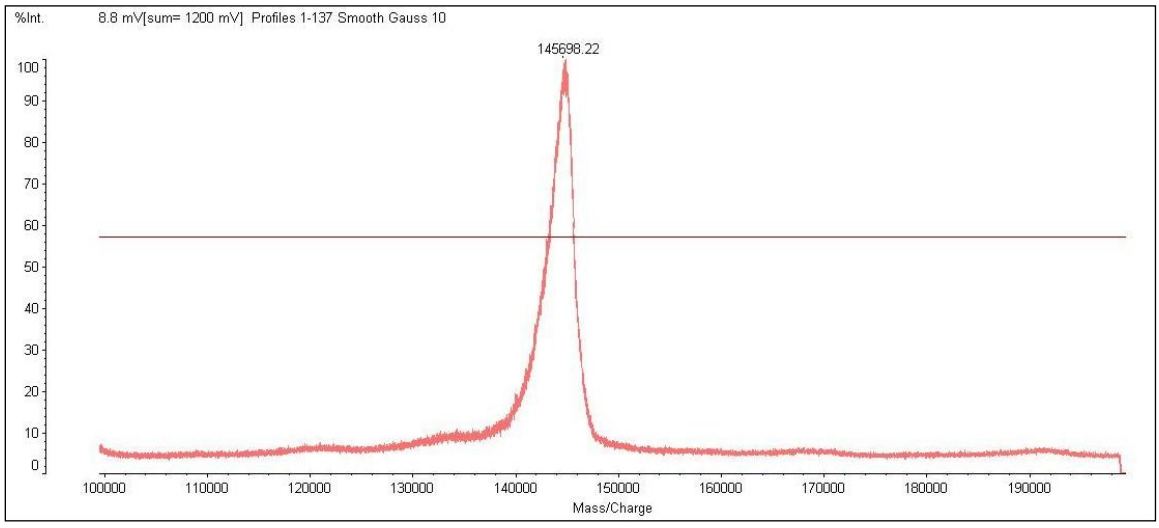


Figure 3. MALDI-TOF data of CMNB Carboxyfluorescein 9EG7 bioconjugate. The MW is 1320 units higher than the unconjugated MAb.

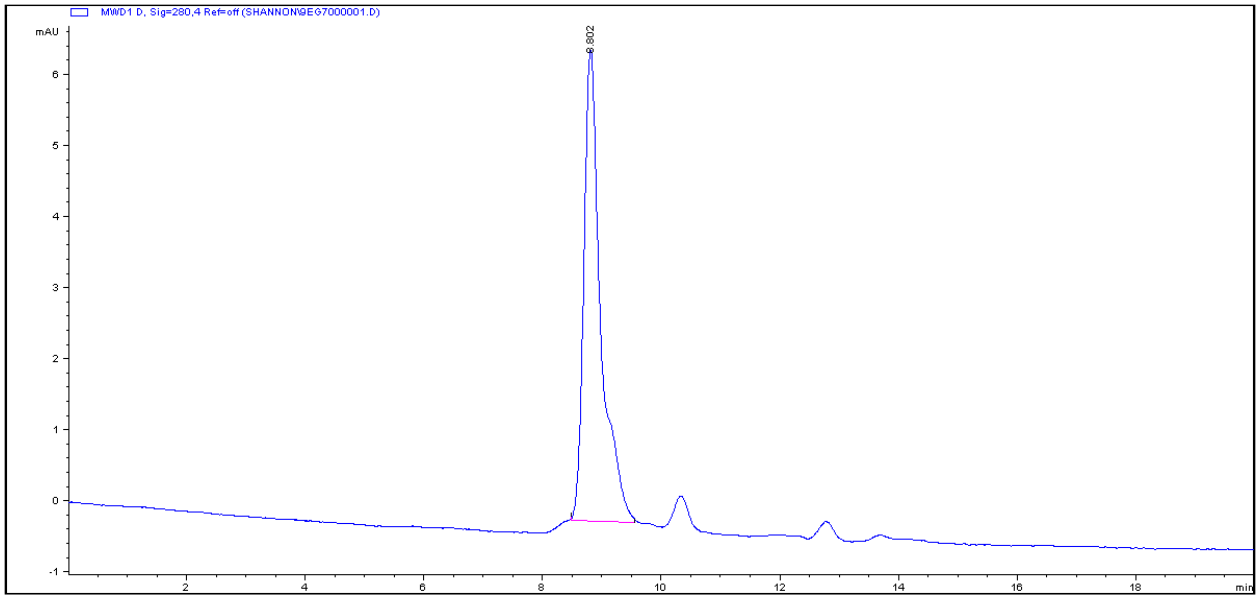


Figure 4. Size-exclusion HPLC of the naked antibody, 9EG7 at 280nm.

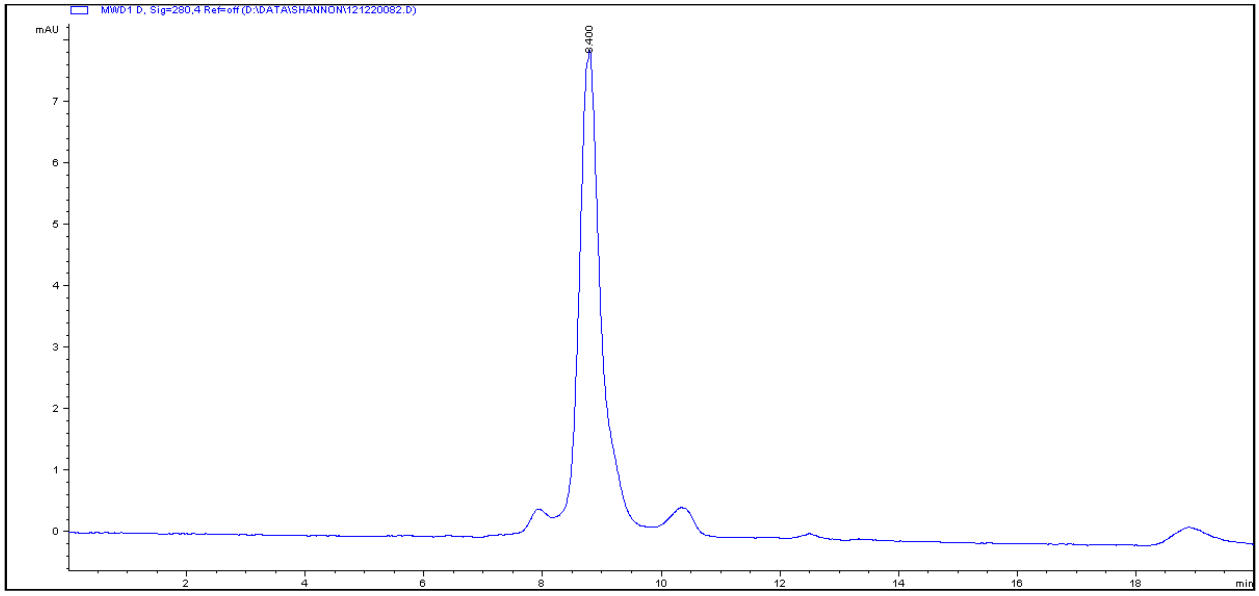


Figure 5. Size-exclusion HPLC of Caged Q-Rhodamine-9EG7 bioconjugate. The R_t of the peak has decreased by ~0.4 minutes.

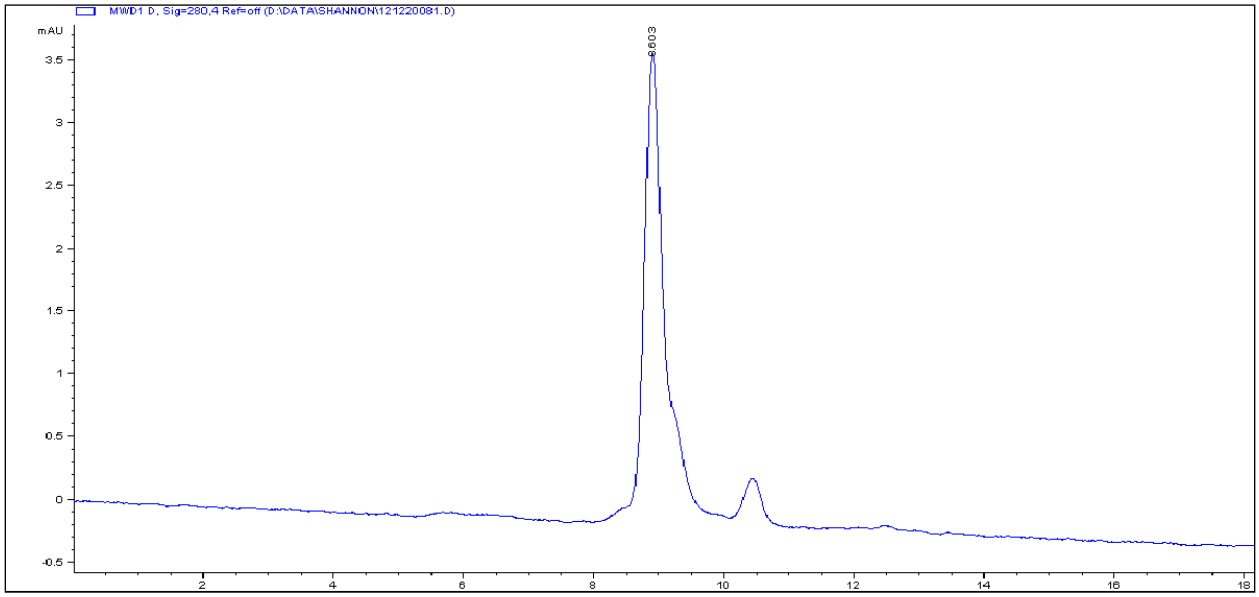


Figure 6. Size-exclusion HPLC of CMNCB Carboxyfluorescein-9EG7 bioconjugate. The R_t of the peak has decreased by ~0.2 minutes.

Experimental

5-Carboxyfluorescein-*bis*(5-carboxymethoxy-2-nitrobenzyl) ether, β-alanine-carboxamide, succinimidyl ester (CMNB-caged carboxyfluorescein, SE) was purchased from Invitrogen (Eugene, OR). 9EG7 antibody was provided by Dr. C. Galbraith.

Buffer exchange was carried out with a Zeba Desalt Spin Column purchased from ThermoScientific (Rockford, IL). Gel filtration purification of the antibody conjugates was performed on an Agilent 1200 Series instrument equipped with a multi-wavelength detector using a Zorbax GF-250 column (9.4 x 250mm, 4µm) with a flow rate of 1mL/min.

MALDI-TOF mass spectra were recorded on a Shimadzu Biotech Axima-CFP Plus Spectrometer. A supersaturated solution of dihydroxybenzoic acid (DHB) (50% CH_3CN, 50% water with 0.1% trifluoroacetic acid) was used as a matrix for MALDI-TOF analysis of the naked and conjugated antibodies.

Double Caged Q-Rhodamine 9EG7 conjugate (1). The antibody (0.5mg/mL, 6mL) was provided in an aqueous saline solution containing 0.09% sodium azide. It was exchanged into 10mM phosphate buffered saline, pH 7.4 by Zeba spin–column (ThermoScientific) followed by lyophilization. To the lyophilized antibody was added 300µL of water to give a final antibody concentration of 10 mg/mL (20.78 nmol). While protecting from light, the double caged Q-rhodamine NHS ester was dissolved in dimethylformamide (DMF) at a concentration of 10mg/mL. To the antibody was then added 9µL (80 nmol) of the Q-rhodamine NHS solution and mixed well. The mixture was allowed to react for 2 hours at room temperature with gentle shaking and then at 4˚C overnight. The conjugated antibody was purified by gel filtration HPLC, affording a clear liquid. R*t* = 9.1 minutes. *m/z* (MALDI-TOF) 145358.29.

CMNB Caged Carboxyfluorescein 9EG7 conjugate (2). The antibody (0.5mg/mL, 2 mL) was provided in aqueous saline solution containing 0.09% sodium azide, and exchanged into 10mM phosphate buffered saline, pH 7.4, by spin-column buffer exchange followed by lyophilization. To the lyophilized antibody was added 100µL of water to give a final antibody concentration of 10mg/mL (6.93nmol). While protecting from light, the CMNB-caged carboxyfluorescein NHS ester was dissolved in dimethylformamide (DMF) at a concentration of 10mg/mL. To the antibody was then added 10µL (100nmol) of the CMNB-caged carboxyfluorescein NHS solution and mixed well. The mixture was allowed to react for 2 hours at room temperature with gentle shaking and then at 4˚C overnight. The conjugated antibody was purified from any excess dye by gel filtration HPLC, affording a clear liquid. R*t* = 9 minutes. *m/z* (MALDI-TOF) 145698.22.

Characterization of bioconjugates. The concentrations of the antibodies were calculated from their absorbance at 280nm. It was determined that there was 36.88µmol of Caged Q-rhodamine 9EG7 conjugate and 13.58µmol of CMNB caged carboxyfluorescein 9EG7 conjugate. However, the UV-Vis spectra gave no supporting data that can be used for the analysis of the ratio of dye molecules to the 9EG7 antibody. The ratio of dye molecules to 9EG7 was calculated from the difference in molecular weights of the conjugated and unconjugated antibodies as determined by MALDI-TOF (Figures 1-3). According to the MALDI-TOF data, there are approximately 0.9 Caged Q-rhodamine molecules per 9EG7 antibody and 1.4 CMNB-caged carboxyfluorescein

molecules per 9EG7 antibody. The purity of the conjugates were obtained by SE HPLC (Figures 5 and 6) and compared to unmodified antibody (Figure 4). It was found for both conjugates that the retention time decreased in the case of caged Q-rhodamine-9EG7 conjugate by ~0.4 minutes and CMNCB carboxyfluorescein-9EG7 conjugate by ~0.2 minutes.

References

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3. Betzig E, Patterson GH, Sougrat R, Lindwasser OW, Olenych S, Bonifacino JS, Davidson MW, Lippincott-Schwartz J and Hess HF. Imaging intracellular fluorescent proteins at nanometer resolution. Science. 2006 Sep 15;313(5793): 1642-5. PMID 16902090